Anti-CRISPRs Oligonucleotides Facilitate Cell Type-Specific Control of Nanoparticle Cas9 Gene Editing

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Abstract

While CRISPR-Cas systems represent a powerful research tool, in order to become clinically viable significant challenges woud have to be overcome. One of the largest issues facing the breadth of CRISPR technologies is the lack of cell type specific gene editing outside of hepatocytes. Controlling the cell type-specific activity of CRISPR-based drugs would enable new Cas9 therapies. Natural anti-CRISPRs can inhibit gene editing, suggesting that synthetic anti-CRISPRs delivered to 'off target' cells could limit undesired gene editing *in vivo*. This report shows that anti-CRISPRs termed inhibitory oligonucleotides (iOligos), which target single guide RNA (sgRNA) via a Rnase H15 independent mechanism, can modulate gene editing in adult mice. By delivering iOligos to hepatocytes, Cas9 hepatocyte editing is blocked, altering the tropism of a nanoparticle so it preferentially edits genes in the spleen. This represents a technology by which it is possible to markedly suppress gene editing in hepatocytes without reducing splenic editing. Synthetic anti-CRISPRs can improve the reach and specificity of Cas9 therapies in adult mammals without improvements in drug delivery vehicles like new nanoparticles.

Introduction

CRISPR-based gene editing systems have already been used to incredible success in research settings and hold incredible promise as potential components of therapeutic systems (*1*). The CRISPR-Cas system is incredibly robust and easy to use which has led to rapid expansion of numerous technologies in genome editing, gene regulation, and even RNA targeting (2). CRISPR-Cas systems exist in nature as prokaryotic immune response mechanisms in a variety of different bacterial species (3). CRISPR-Cas systems are capable of highly specific nucleic acid targeting and cleavage. All these systems rely on CRISPR RNA (crRNA in nature or guide RNA (gRNA) in experimental systems. The spacer, or interchangeable portion of the guide RNA, is complementary to the targeted sequence and hybridizes to the targeted sequence. This hybridization guides the Cas protein which cleaves the target sequence, resulting in site-specific editing at nearly any location (2). This specificity and accuracy is more efficient, and importantly relies on nucleic acid pairing rather than protein-DNA binding which makes the system incredibly easy to implement. Development and improvements in type II CRISPR-Cas9 systems led to an explosion in the use of this powerful technology in research as well as attempts at translating the technology to for clinical applications (4).

However, the clinical usefulness of CRISPR-Cas systems has been limited at best due to off target effects and the lack of cell specific delivery systems. In animal models local or site specific CRISPR therapies rely on local delivery such as direct injection at the target site (5). This site-specific delivery methodology may work for researching the effectiveness of gene editing systems but limits their clinical translatability. While systemic delivery has been attempted there are limits in the efficacy of this approach as well. First these systemic delivery systems have basically been limited to editing in hepatocytes (6, 7). Systemic delivery disproportionately traffics

these molecules to the liver, resulting in unbalanced editing in hepatocytes and limited editing elsewhere. This is a serious problem for the translation of CRISPR-Cas systems, but not a problem specific to them. Many nanoparticles disproportionately and preferentially target the liver (8). While delivery to other organs does occur accumulation of clinically relevant and predictable doses without overdosing the liver has remained a challenge. Nanoparticles that deliver or target other organs, face a number of barriers including endothelial barriers, cellular membranes, and the endosome.

Great efforts have been made however to develop nanoparticles that are capable of cell type specific drug delivery. By varying the chemical makeup of a nanoparticle as well as its size and charge scientists are able to optimize nanoparticle design to the target cell type (9). Many nanoparticles have even been conjugated with specific targeting ligands that bind to receptors on the target cell in order to further increase their targeting capability. Despite the numerous variables that can be changed to affect targeting and the countless number of nanoparticles that have been developed with this in mind, off-target hepatocyte delivery remains an unsolved problem (8). The current methods by which nanoparticles are developed and optimized has failed to produce a nanoparticle that can avoid hepatocytes and specifically target a new cell type. However, a reliable and programmable way to enable cell type specific gene editing outside of hepatocytes would be doubtless a major advancement for CRISPR therapeutics and for research purposes.

Rather than working to develop nanoparticles that avoid hepatocytes, an alternative would be to block the effectiveness of a drug, specifically in hepatocytes. This would make their preferential delivery advantageous, providing the opportunity to preload the liver with a molecule that inactivates the drugs later delivered to both hepatocytes and the target cell type. Development

of protein-based anti-CRISPRs that reduce gene editing in vitro (10) led to the hypothesis that anti-CRISPR oligonucleotides could also reduce Cas9 editing.

The preference for oligonucleotide based anti-CRISPRs over the previously described protein-based models that interfere with the RNP (ribonucleoprotein formed by conjugation of Cas9 and sgRNA) came from several reasons. Oligonucleotides have low toxicity and high tolerance in humans and animal models, oligonucleotides can be chemically modified for stability, and critically there already exists clinically approved lipid nanoparticles (LNPs) that deliver oligos to hepatocytes (11). These nanoparticles allow the implementation of the previously described methodology that turns hepatocyte preference from a disadvantage to an advantage. Here by pre-delivering synthetic oligonucleotide anti-CRISPRs (interfering oligos or iOligos) to hepatocytes there was a significant reduction in gene editing, which will facilitate targeted genetic editing outside the liver.

Methods and Materials

Oligonucleotide & siRNA Synthesis: iOligos were purchased from Integrated DNA Technologies (IDT). Many Oligos were chemically modified with 2'O-methyl ribose and phosphorothioate modifications to increase stability, reduce immunogenicity, and increase affinity between the oligo and target RNA (*18*). siRNAs (siLuc, siICAM2, siGFP, hm-sgGFP) and sgRNAs (sgGFP, sgICAM2) were purchased from AxoLabs. Messenger mRNAs were either purchased from Trilink Biotechnologies (GFP, Cas12a) or synthesized as described below (SpCas9).

Cell Culture: *In vitro* experiments were performed using Immortalized Aortic Endothelial Cells (iMAECs), or IMAECs stably transduced with CAG-SpCas9-EGFP. IMAECs were cultured in EGM-2 Growth Media (Lonza). HEK293 cells were cultured in DMEM/F-12 50/50 media (Corning) supplemented by 10% (v/v) FBS (VWR) and 1% (v/v) penicillin-streptomycin (ThermoFisher Scientific). Cells were seeded in a 24-well plate at a density of 50k cells / well. DNA was isolated using 40 μL of QuickExtract (EpiCentre).

TIDE: Indels were measured by Tracking Indels by Decomposition. Briefly, a ~600-800 nucleotide amplicon surrounding the sgRNA-binding sequence was amplified using PCR. This amplicon was then Sanger sequenced by Eton Biosciences.

Nanoparticle Formulation: Nanoparticles were formulated using a microfluidic device. Nucleic acids (siRNA and DNA barcodes) were diluted in citrate buffer while lipid-amine compounds (cKK-E12, 7C1), alkyl tailed PEG, cholesterol, and DSPC were diluted in ethanol. PEG, cholesterol, and DSPC was purchased from Avanti Lipids. Citrate and ethanol phases were combined in a microfluidic device by syringe pumps.

Animal Experiments: All animal experiments were performed in accordance with the Georgia Institute of Technology's Physiological Research Laboratory (PRL) animal care and services policy. C57BL/6J (#000664) and constitutive SpCas9 (#026179) mice were purchased from The Jackson Laboratory and used between 5-12 weeks of age. The nanoparticle concentration was determined using NanoDrop (Thermo Scientific).

Cell Isolation & Staining. Cells were isolated 72 hours after injection with LNPs unless otherwise noted. Mice were perfused with 20 mL of 1X PBS through the right atrium. Tissues were finely cut, and then placed in a digestive enzyme solution with Collagenase Type I (Sigma Aldrich), Collagenase XI (Sigma Aldrich) and Hyaluronidase (Sigma Aldrich) at 37oC at 550rpm for 45 minutes. The digestive enzyme for spleen included Collagenase IV. Cell suspension was filtered through 70μm mesh and red blood cells were lysed. Cells were stained to identify specific cell populations and sorted using the BD FacsFusion in the Georgia Institute of Technology Cellular Analysis Core. For *in vitro* experiments, a BD Accuri C6 was used. The antibody clones were used: anti-CD31 (BioLegend, 390), anti-CD45.2 (BioLegend, 104), We defined cell populations in the following manner: Splenic endothelial cells (CD31+CD45-), and hepatocytes (Liver, CD31-CD45-).

Cas9 Intracellular Staining: To determine Cas9 protein translation, the FoxP3 / Transcription Factor Staining Buffer kit (Tonbo Biosciences) was used to fix and permeabilize the cells. Anti Cas9 primary antibody (Biolegend, 7A9) and AlexaFluor 647 anti-mouse IgG1 (Biolegend, RMG-1) was used to quantify Cas9 protein using a BD Accuri C6 flow cytometer.

Results & Discussion

It was first necessary to determine whether small chemically modified oligonucleotides could act as an anti-CRISPRs at all. 4 different iOligo sequences were developed to match different sections of the conserved region of sgRNA (12). 25 initial experiments were performed in immortalized aortic endothelial cells (iMAECs) (*13*). These cells were first transduced with lentivirus to stably express Cas9 (hereafter termed Cas9-iMAECs).

Lipofectamine 2000 was then used to transfect iOligos into Cas9-iMAECs; 4 hours later, the same cells were transfected with sgRNA targeting ICAM-2 (sgICAM-2). Seventy-two hours later, genomic DNA was isolated from the cells and quantified insertions and deletions (indels) using Tracking of Indels by Decomposition (TIDE) (*14*). Compared to a scrambled oligonucleotide which acted as a control, all four iOligos reduced Cas9-mediated indels, suggesting the iOligos can block sgRNA activity in murine cells. The 4th iOligo, iOligo-D which was targeted to the 3' end of the sgRNA, was the most effective in reducing indels. All four iOligos however, reduced indel formation (**Fig. 1A)** in a dose-dependent way (**Fig. 1B**) in Cas9-iMAECs. iOligoD (now just iOligo) was the most effective in reducing indels and was selected for all the future experiments.

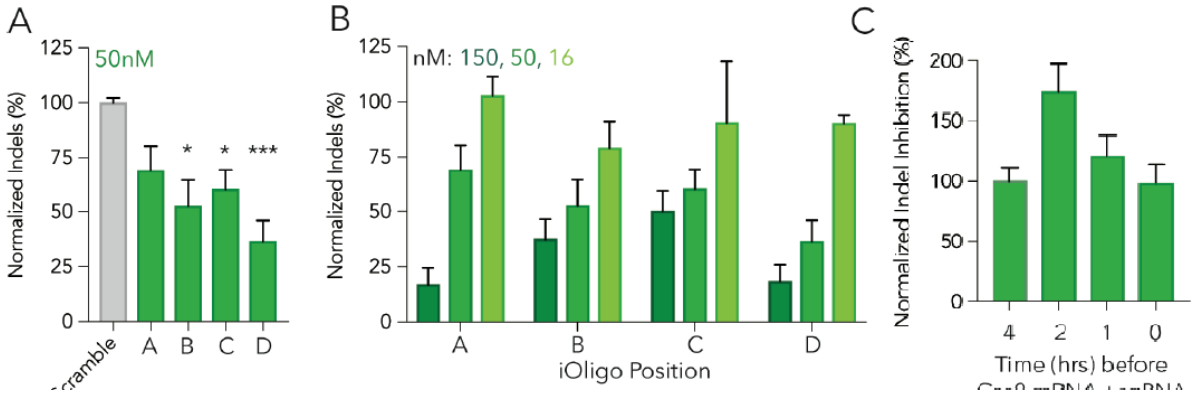

*Figure 1**(A)** indel percentage in Cas9 expressing cells following treatment with iOligos or control; iOligos prevent indel formation in cell culture *p<0.05, ***p<0.001, One-Way ANOVA. **(E)** each of the 4 iOligos inhibit indel formation in a dose dependent manner. **(C)** Normalized indel inhibition based on the amount of time between iOligo treatment and Cas9 mRNA+sgRNA in normal cells.*

To confirm these results, which were all generated in Cas9-iMAECs, additional experiments were performed to determine whether iOligos maintained functionality when Cas9 was delivered transiently via mRNA. First iOligos were transfected at a dose of 16nM, then wildtype iMAECs were transfected with 300ng Cas9 mRNA and 16 nM sgICAM-2. As expected, iOligos reduced indel formation. When the time between iOligo administration and Cas9 administration was varied, it was found that iOligo efficacy was most effective 2 hours prior to the delivery of mRNA and sgRNA (**Fig. 1C).** These experiments suggest that chemically modified, small oligonucleotides are capable of blocking Cas9 activity *in vitro.*

Next, it was tested if this promising activity held up *in vivo.* So, experimentation was moved from cell culture to several models of adult mice to determine whether iOligo could still regulate gene editing in animal models. The first target for this reduction was hepatocytes. Hepatocyte targeting lipid nanoparticles were formulated by mixing $C_{14}PEG_{2000}$, cholesterol, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and the ionizable lipid cKK-E12 (*22*) in a microfluidic device as previously described (*15*). This specific Lipid nanoparticle delivers oligonucleotides to hepatocytes *in vivo (16).* Hepatocyte targeting LNPs were formulated to carry iOligo as a load and additional LNPs of the same formulation were made to carry the scrambled sequence as a control. Again, this scrambled sequence replicates the molecular weight and any potential changes to delivery or distribution while being an inactive sequence. hepatocyte targeting LNPs were also formulated to carry sgGFP, sgRNA that was complimentary to a portion of the gene encoding the production of GFP (green fluorescent protein). In all three cases, small, stable LNPs with low polydispersity were formed.

CRISPR-Cas9 knockin mice that express SpCas9-GFP under a ubiquitous CAG promoter (*17*) were injected with either iOligo or the control oligo. Two hours later, the same mice were injected with sgGFP. Five days later the mice were sacrificed, and hepatocytes were isolated from the liver (CD31-CD45-) using fluorescence activated cell sorting (FACS) and GFP protein expression and indels were quantified and recorded (**Fig 2 A)**.

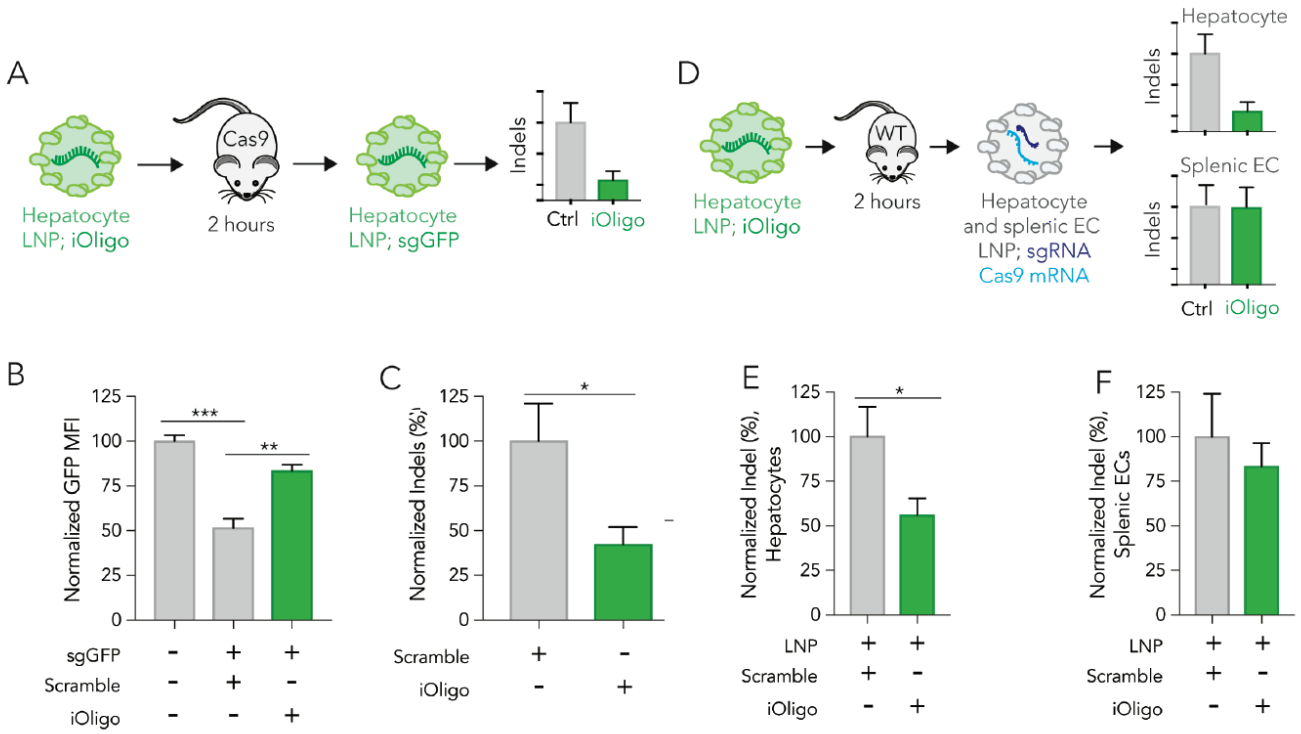

*Figure 2(**A**) Mice that express spCas9 were pretreated with iOligos delivered by a hepatocyte targeting LNP; two hours later the same LNP delivered sgGFP. iOligos were expected to downregulate indel formation. (**B**) Normalized GFP MFI in hepatocytes decreased in mice pre-treated with a control oligo relative to iOligos. **p<0.01, ***p<0.001, One-Way ANOVA. (**C**) Normalized indel percentage in hepatocytes also decreased in mice pre-treated with iOligo relative to mice treated with the control oligo. (**D**) Wild-type mice were pretreated with iOligos delivered by hepatocyte targeting LNP and two hours later the same mice were treated with LNPs carrying Cas9 mRNA and sgICAM-2. iOligos were expected to decrease indel formation in hepatocytes without impacting indels in splenic endothelial cells. (**E**) normalized indel percentage in hepatocytes in mice pre-treated with iOligo and control oligo, *p<0.05, Two-tail T-Test. (**F**) Normalized indel percentage in splenic endothelial cells did not significantly change in mice pretreated with iOligo relative to control.*

Compared to control mice injected with PBS, GFP expression in mice injected with control oligo and sgGFP was reduced by 50% as measured by mean fluorescent intensity (MFI) (**Fig. 2B**). GFP expression in mice treated with iOligo and sgGFP was statistically higher, suggesting that iOligo blocked sgGFP gene editing in Cas9 mice (**Fig. 2B**). Indel percentages decreased by 58%

in iOligo treated mice, relative to mice treated with the control oligo (**Fig. 2C**), suggesting the effect was Cas9-mediated.

While the results of the above indicate that iOligo can suppress genetic editing in the liver, it remained to be tested whether iOligos would have deleterious effects on genetic editing in other organs. In order to test this next step iOligos were tested in wildtype C57BL/6 mice, which are generally considered more clinically relevant than transgenic Cas9 mice. Wildtype C57BL/6 are more genetically representative of how CRISPR based genetic editing systems may eventually be translated clinically. For the wildtype C57BL/6 injections the iOligo or scramble control were formulated into the hepatocyte-targeting LNP, then administered intravenously to wildtype adult mice (**Fig. 2D**). Two hours later, the mice were injected with LNPs carrying Cas9 mRNA and a chemically modified sgRNA targeting ICAM-2 (*17*). The second injection utilized recently reported LNPs that deliver Cas9 mRNA and sgRNA to both hepatocytes and splenic endothelial cells (*25*). So while the sgRNA and Cas9 mRNA were delivered to splenic endothelial cells and hepatocytes, the iOligos were only delivered to hepatocytes. Once again, the mice were later sacrificed and hepatocytes and splenic endothelial cells ($CD31^{+}CD45^{-}$) were isolated using FACS. The results indicate that pre-delivery of iOligos to hepatocytes resulted in a statistically significant reduction in hepatocyte indels (**Fig. 2E**), but not splenic endothelial cell indels (**Fig. 2F)**. This suggests that iOligo delivery to hepatocytes can reduce hepatocyte editing without reducing editing in other cell types within the same animal.

Conclusion & Future Work

The data suggests that iOligos are able to help facilitate cell type specific control of Cas9 gene editing. While the percentage of indels in hepatocytes remained higher than preferable for clinical applications, this tool represents remarkable potential. First, as a first run it is not only possible but very likely that iOligo can be further optimized in future work. Potentially, one could improve the potency of iOligo by altering its chemical modifications and sequences to increase stability and promote higher affinity binding. Ideally, these improvements in structure and modification, along with an optimized dosing schedule (to maximize the effective time window of iOligo) will help to reduce hepatocyte gene editing efficacy to undetectable levels. An alternative method by which this system could further be improved is with the combination of other therapies such as siRNA. Cas9 mRNA and sgRNA are often co-delivered in the same LNP. Since iOligos target the sgRNA and not the production of Cas9 its possible that iOligos could be co-delivered with siRNA designed to degrade Cas9 mRNA further driving down editing.

This work could help enable the development of systemic, non-hepatocyte Cas9 therapies by removing an important nanoparticle design criterion. Historically, a nanoparticle would need to deliver Cas9 mRNA + sgRNA exclusively to a new target cell; now a nanoparticle that delivers Cas9 mRNA + sgRNA to a desired target cell type and hepatocytes could still be sufficient for cell type specific editing. This gives a much broader range to the type and number of nanoparticles that can be design and discovered and still have clinical significance. Rather than avoiding problematic cell populations that are too prone to targeting and off target effects this technology uses that property to its advantage to provide editing specificity and opens the door to a broader array of potentially viable nanoparticles and editing techniques.

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